Recent Research of Endocrine Disrupters Testing of Amphibians in Japan

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Thank you for the introduction. My research concerns amphibians, particularly water-electrolyte metabolism of anuran amphibians and related endocrine. Japan has begun research of a test method and screening of endocrine disrupters using anuran amphibians since this summer, primarily conducted by the OECD and Ministry of the Environment. Today, I would therefore like to talk about the first OECD conference of amphibian experts, subsequent activities of Japan and how they are progressing.

Amphibians are good specimens for research of development and metamorphosis, as well as sex differentiation, physiology, etc. They are also a group of animals with which we are very familiar.

Amphibians were the first vertebrates to appear on land over 300 million years ago. In the past 10 to 20 years, populations of more than 200 species of amphibians throughout the world have been drastically reduced, and it is thought that about 20 species have altogether become extinct. Reasons for such a dramatic drop in populations include changes in the global environment, contamination by pesticides, and spread of fatal pathogenic bacteria. Therefore, it can be also said that Amphibians play a similar role to warn us about global environmental pollution as canaries in detecting safety of mines.

Despite the fact that amphibian populations have dropped sharply on the global level, study and research of endocrine disrupters and amphibians is behind the times, with the exception of a few researchers. At the first meeting of the EDTA study committee in 1998, it was announced that there was no method of testing (endocrine disrupters) using amphibians. The need for testing using amphibians was brought up at the fourth meeting in 2000, and a meeting of experts concerning a method of testing endocrine disrupters using amphibians was held at the Paris headquarters of the OECD in April 2001. The participating countries are Denmark, France, Germany, Italy, Japan, Sweden, Switzerland, the United-Kingdom, and the U.S.A. The items given here were decided in this meeting.

Many amphibians pass their juvenile period in the water. After going through metamorphosis and becoming adults, they begin to live on land. Morphological and functional transformations occur during the process of metamorphosis, and thyroid hormone has an important function during this process. Thyroid hormone is regulated by thyroid stimulating hormone (TSH) secreted from the hypophysis, the pituitary gland, and thyrotropin releasing hormone (TRH) from the hypothalamus. Therefore, screening of the hypothalamus-pituitary-thyroid axis has particularly been proposed for tests involving endocrine disrupters using amphibians. The various countries therefore need to work on this.

This shows the research that is scheduled to be conducted in Japan. In addition to research on hypothalamus-pituitary-thyroid axis, research of the problem of sex differentiation in amphibians is considered to be extremely important.

This figure shows target chemicals for research of endocrine disruption of the hypothalamus-pituitary-thyroid axis using amphibians announced at the meeting of experts on the subject. T_3 and T_4 are the positive controls and propylthiouracil is the negative control. The other chemicals are those for which effect on the thyroid gland have been reported in literature on the subject. Octachlorostyrene, which required early study of its endocrine disrupting effect, began to be studied in 2000. The chemical has been listed by the "Strategic Programs on Environmental Endocrine Disrupters ’98 (SPEED ’98)" as byproducts of organochlorine compounds in Japan. I would also like to report the results of our current research of bisphenol A (BPA), a raw material used in the production of resin that is reported to have a sex hormone-like effect.

This figure shows the results of a study of acute toxicity of octachlorostyrene on Xenopus laevis tadpoles. After being treated with octachlorostyrene for 72 hours, the LD50 of ST48 tadpoles was approximately 140 μg/l. This is hematoxylin and eosin stained liver tissue of tadpoles treated with octachlorostyrene for 24 hours by means of a light microscopy. Compared with the control group, fatty degeneration of liver cells is more pronounced in the group treated with 100 μg/l and lymphocyte infiltration is also observed. Atrophy of hepatic cell cords, deciduation of hepatocytes, reduction, fatty degeneration, lymphocyte infiltration and necrosis were observed in the group treated with 1000 μg/l. This individual died of liver failure. Although extension of the sinus venosus due to insufficient circulation was observed in these individuals, effect on endocrine glands such as the thyroid gland was not evident under a light microscope. With the advance of developmental stages, resistance to toxicity of octachlorostyrene was observed to increase.

Here are the results of screening of octachlorostyrene conducted using the Xenopus Metamorphosis Assay method (XEMA), which is proposed as the ring test by groups in Germany, etc. The vertical axis indicates developmental stage based on the Newkoup stage table. The horizontal axis represents treatment time with the concerned solution. Tadpoles at ST 48-49 were used and grew to ST57-58 four weeks later. The XEMA method was used up to four weeks after treatments, and we continued the study until completion of metamorphosis. The *Xenopus laevis* tadpoles were fed the same food and kept in the same aquatic environment (0.025 g/l seawater) as was used for the ring test in Germany, etc., for a period of four weeks, at which time the tadpoles steadily grew to approximately ST58. In case of ethylene thiourea treatment, the tadpoles remain ST53. As for the other groups, starting from the group at a notable developmental stage, the group order was the groups treated with 50 μg/l of octachlorostyrene, 25 μg/l, T4, 1 μg/l, control, and groups treated with 10, 1 and 5 μg/l of octachlorostyrene. No significant difference from the control groups was observed. T4 concentration here was 1 μg/l. With T4 concentration 8 μg/l treatment, the specimens had already developed to ST54 at the first week. Development of fore legs and transformation of the skull were observed, and metamorphosis of the body progressed, although the body was meager.

These are the results of a study of the effects of octachlorostyrene by Xenopus Metamorphosis Assay. The vertical axis shows the results of the overall body length and tail length in tadpoles from each group, which were photographed every three days with a digital camera and measured using a computer. As a result of the assay, overall body length of the group treated with 50 μg/l of octachlorostyrene tended to be much greater than for the control groups. Growth was slower at lower doses of octachlorostyrene and development tended to be poor.

This figure shows the results of a study of the effect of BPA on regression of the tails of *Rana rugosa* tadpoles treated with T_3, which is a type of thyroid hormone. First of all, the tadpoles that have reached stage 11 (T.K.) are kept in dechlorinated tap water (untreated and group 3) or dechlorinated tap water to which BPA has been added (groups 1 and 2) for a period of seven days. Next, tadpoles belonging to groups 2 and 3 are treated for a period of 24 hours in 5×10^{-8} M of T_3 in the previously described environment. Finally, after T_3 treatment, the tadpoles are once again kept in the initially described environment for a period of four days. When measuring tail length, the start of T_3 treatment was considered to be day zero. Change in tail length was indicated to day zero as 100%. As the graph clearly shows, regression of the tail due to T_3 treatment was observed in group 3. Regression of the tail however was not observed in group 2, although it was treated with T_3 as well. BPA therefore was observed to inhibit regression of the tail due to T_3. This therefore suggests that BPA inhibits the effect of thyroid hormones.

Proteome is an effective means of closing in on various biological processes and mechanisms. After completing the tests mentioned a little while ago, tail and liver samples were taken from individuals

in each group, the protein was extracted and two-dimensional electrophoresis was conducted. These are the results of two-dimensional electrophoresis of protein extracted from the tail. The spots indicated by orange are the spots where expression increased in comparison with the electrophograms of untreated individuals. The pink spots are spots where it decreased in comparison with the electrophograms of untreated individuals.

Thus it was found that expression of protein varies for each group. The next spot indicates an interesting change.

As for this spot, expression increases in the individuals of group 3 (T_3 treated) only. There is no change in the individuals of group 2 in this spot, although they were also treated with T_3. This therefore suggests that this spot is directly or indirectly related to inhibition of tail regression by BPA.

This figure shows electrophoresis of protein extracted from the livers of individuals of each group following completion of the previously described experiment. Just as before, the orange spots are the spots where expression increased in comparison with the two-dimensional electrophograms of untreated individuals and the pink spots are spots where it decreased.

Just as with that tail, therefore, we found that expression of protein in the liver varied largely from group to group. The next spot indicates an interesting change.

The spots indicated in 99L are spots where expression increased for individuals of group 3 only. No change was observed in these spots for the individuals of group 2 although they were treated with T_3. This therefore suggests that these spots are protein that is directly or indirectly related to inhibition of tail regression by BPA. The spots shown in 52L on the other hand are spots where expression increased for individuals of groups 2 and 3. No change was observed for the individuals of group 1 and the untreated group in these spots. In other words, these are the spots where expression uniquely increased due to T3 treatment. This therefore suggests that these spots are protein that is related to regression of the tail but is not affected by BPA. It is therefore hoped that proteome analysis will explain the mechanism by which endocrine disrupters act and will be able to find markers that detect endocrine disrupting effect.

Next is development of a method of screening endocrine disrupters using transgenic frogs. Thyroid hormones are molecules that control metamorphosis of amphibians, and the nuclear receptors that transmit the signal have also been identified (TRα/β). TR forms retinoid X receptors (RXR) and heterodimers and controls gene transcription by bonding with DNA sequences called thyroid hormone responsive sequences, or TRE, under existence or nonexistence of thyroid hormone (T_3).

We therefore prepared a plasmid (pEGFP/xTRβA1) inserted with an enhanced green fluorescent protein (EGFP) gene at the bottom of the sequence near the transcription starting point 1.6 kbp (-1300 to +300) of a *Xenopus* TRβA1 gene equipped with TRE. We then created a transgenic frog using the plasmid.

This figure shows the procedure by which the transgenic frog was created. Sperm nuclei from the sperm of a male frog is first adjusted and is then mixed with the plasmid. The transgenic frog is then created by transplanting the sperm nuclei in an unfertilized egg from a female frog.

We assessed responsiveness of transgenic tadpoles and frogs in which the plasmid (pEGFP/xTRβA1) was introduced to T_3. First I'd like to talk about transgenic juveniles. As shown in the figure on the left, at NF stage 51, T_3 was added to the water in which the juveniles were kept so that it became 1 nM, and then we monitored it for expression of EGFP. On the third day after the experiment began, an increase in fluorescence was observed, and an even stronger fluorescence was confirmed on the fifth day. Prominent response was exhibited by the limb buds and nervous system. As shown in the figure on the right, T_3 was added to the water in which the transgenic frogs were kept so that it became 1 nM, and then we monitored it for expression of EGFP with time. Just as with the experiment with juveniles, an increase in fluorescence was observed on the third day after the experiment began, and an even stronger fluorescence was confirmed on the fifth day. The results of adding chemicals to the water in which the

specimens were kept and then measuring intensity of fluorescence suggest the endocrine disrupting effect of the signal transmission system via TR can be assessed.

Next we obtained F_1 by waiting for sexual maturation of the groups of transgenic frogs we created and then mating them with wild frogs. *Xenopus laevis* lay eggs from several hundred to a thousand eggs at once. If you are able to obtain the second generation (F_1) that received foreign genes, it enables you to assess various chemical groups using transgenic frogs. When we tested responsiveness by adding T_3 to the water in which F_1 individuals that had reached stage 51 were kept so that it became 1 nM, just as with the first generation of transgenic frogs, an increase in fluorescence was observed on the third day after the experiment began, and an even stronger fluorescence was confirmed on the fifth day. Prominent response was exhibited by the limb buds and nervous system. The experiment clearly showed that responsiveness of the first generation of transgenic frogs to T_3 is passed on to the next generation. Transgenic frogs are therefore thought to be a useful tool for assessing endocrine disrupting effect of signal transmission system via TR.

According to the distribution study conducted by the Ministry of the Environment every few years using a mesh map for each of the prefecture of Japan, fluctuation in distribution and number of individuals are not sufficiently known. The area of Japan where deformed frogs were observed in 1999 and 2000 is encircled in red in the figure. With certain exceptions, the causes are unknown.

This is a microscopic photograph of a hermaphrodite *Rana ornativentris* reported in 1964. With testis on the right and the ovary on the left, this individual has well-developed gonads. On the right is a photograph of testis-ova found in the testis of various types of Japanese frogs in 2000. In case of frogs, egg cells have been observed in testis busing spermatogenesis. The existence of such hermaphrodites and testis-ova in the natural environment was first reported in the West in the 1920s and in Japan around 1950s. It is however still unknown if this phenomenon is a distinguishing characteristic of sex differential in anuran amphibians.

This is the testing method to screen chemicals for estrogenic effect using *Xenopus laevis* developed by Doctors Hanaoka and Mikami of Gunma University. The sex chromosomes of normal frogs are female hetero ZW type. If male frogs are treated with esrtrogens, they mature into females regardless of their genetic sex. If these sex-reversal females are mated with males, the next generation of tadpoles will be male individuals with pure ZZ sex chromosomes. The sex of individuals raised in waters containing chemicals suspected of being endocrine disrupters is morphologically monitored after completion of metamorphosis. This allows the change of males to females by chemicals substances to be expressed quantitatively.

This is a photograph taken through an optical microscope showing formation of testis-ova by treatment with female hormones.

These are the results of treating male *Xenopus laevis* tadpoles in water containing female hormones or DES until metamorphosis is complete. This method enables us to express quantitatively that males were changed to females in both the groups treated with estradiol-17β and the dietilstilbesterol group.

Amphibians, especially frogs, might be good models for research of certain endocrine disrupters from life history. We therefore would like to do the following:

1. Continue the screening test for effect of endocrine disrupters on amphibians.
2. Conduct screening on Japanese frogs in addition to XEMA.
3. Continue to conduct field study and analysis of amphibians (distribution, accumulation of chemicals in the body, hormone level).

These people assisted me in preparing this presentation. I'd therefore like to take this opportunity to thank them for their help.

Q&A

Koëter: Thank you. Is there a question from the audience? Yes, please.

Pickford: Dan Pickford, AstraZeneca. Thank you for a very interesting talk. I was interested by the data on bisphenol A inhibition of tail absorption in . At our lab we conducted a chronic larval study with *Xenopus laevis* and bisphenol A at concentrations spanning the concentrations you used, but without exogenous T_3. We saw no effect on larval development.
Do you think that the discrepancy here is due to differences in species, or maybe the fact that you are using an exogenous T_3 stimulus as opposed to normal development?

Uchiyama: Well, I do not know. I think we had better try and check more, other species. Also, you know we did not check the *Xenopus laevis* yet.

Pickford: Thank you.

Koëter: Thank you. Any more questions? I think we came close to the time that we were allotted.